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Microbial metabolites profile during *in vitro* human colonic fermentation of breakfast menus consumed by Mexican school children

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ABSTRACT

The nutrition transition promotes the development of childhood obesity. Currently, Mexico is affected by this serious public health problem. The nutritional and functional characterization of a whole menu has a number of advantages over the study of single nutrients. Since breakfast is considered the most important meal of the day, this study aimed to evaluate the metabolite profile produced by *in vitro* human colonic fermentation of the isolated indigestible fraction (IF) from three different Mexican breakfast (M-B) menus (Modified "MM-B", traditional "TM-B", and alternative "AM-B"), previously identified as commonly consumed by Mexican schoolchildren in Nayarit State, Mexico. The M-B's consist of egg, corn tortilla, beans (higher in TM-B), sugar and chocolate powder (higher in AM-B) and milk, combined in different proportions. The IF in all breakfasts was about 4.7–5.6 g/100 g FW, with a relatively high content of protein (≈21%), which might have negative physiological implications. Fermentation of IF from TM-B resulted in the largest pH decrease after 72 h (pH = 6.07), with a low short chain fatty acid (SCFA) production (0.75 to 47.23 mmol/L), but greater relative concentration of other fatty acids (FA) (C7, C8, C9). Besides, 55 volatile compounds were detected in the fermentation media by SPME-GC-MS and three principal components (PC) were identified. PC1 was influenced by SCFA production, low FA esters production (<8C), and low volatile organic acids production. PC2 was influenced by the decrease in pH and an increase in antioxidant capacity ($p < 0.0001$). These results suggest that the production of different metabolites in the luminal medium may affect the pH and antioxidant status in the colon. Fermentation of IF from TM-M, assessed after 48 and 72 h, showed the highest correlation for PC2; the metabolic pattern registered for this IF maybe considered beneficial.

1. Introduction

Childhood obesity has been associated with numerous negative health and psychological outcomes. It has been explained as consequence of globalization-related changes in diet and lifestyle that promote positive energy balance (Malik, Willett, & Hu, 2013). Most dietary guidelines agree on the importance of a correct distribution of calories and nutrients ingested throughout the day, which is achieved principally by a regular meal consumption of breakfast, lunch, and dinner (US-DHHS and USDA, 2015). In Mexico, obesity epidemic among schoolchildren has resulted in federal and state policies that aim to improve school food environments (Levy et al., 2012). It is often stated that breakfast is the most important meal of the day, and the association between breakfast consumption and its nutritional quality is related with a reduced risk to develop metabolic syndrome (Odegaard et al., 2013). However, taking into account that nutrients or foods are rarely eaten isolated, studies of whole menus or dietary patterns have advantages over the single-nutrient or single-food approach, as they take into account the occurrence of synergistic or antagonistic biochemical interactions among nutrients, as well as the existence of different food sources of the same nutrient (Barbaresko, Koch, Schulze, & Nöthlings, 2013).

Abbreviations: AM-B, alternative Mexican breakfast; AOX, antioxidant capacity; CGC/MS, gas chromatography–mass spectrometry; DP, dietary pattern; FA, fatty acid; FW, fresh weight; HCA, Hierarchical cluster analysis; HS-SPME, headspace solid-phase microextraction; IF, Indigestible fraction; MM-B, modified Mexican breakfast; PCA, Principal component analysis; SCFA, short chain fatty acid; TM-B, traditional Mexican breakfast.

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Most available information on breakfast dietary patterns (DP) and health in schoolchildren is derived principally from epidemiological studies (Karatzi et al., 2014). However, information is scare about the composition of whole menus identified in different DP. Probably, the combination of epidemiological and *in vitro* analysis of meal menus may contribute to a better understanding of the effects that food can exert on health. The fermentative processes that take place in the human large intestine have raised much interest in the last decades. Different fermentation-derived metabolites have important physiological activity at both colonic and peripheral levels (Flint, 2016). In this work, we evaluated the metabolites profile obtained after *in vitro* colonic fermentation of the indigestible fraction (IF) isolated from three breakfast menus consumed by Mexican schoolchildren in Nayarit State, Mexico. Particular attention was paid to metabolites associated with changes in pH and antioxidant capacity (AOX). Chemical composition, extractable and non-extractable polyphenols content, and AOX of the isolated IFs were also evaluated.

2. Material and methods

2.1. Preparation of breakfast menus

Data sources on DP and food frequency consumption at breakfast were obtained from a nutritional survey carried out on eleven public schools in Tepic (Nayarit State, Mexico). The study, being reported elsewhere, revealed no clear-cut relationship between overweight-obesity status of school children and diet types, although the macronutrient intake profile was dependent on the type of diet consumed (Zamora-Gasga et al., in press). For this reason, frequently consumed foods in each DP were used to create three breakfast menus, which comprised the following foods: a) Modified Mexican breakfast (MM-B) (≈394 g total weight, 295 Kcal): one scrambled egg, three corn tortillas (90 g), chocolate milk (264 mL whole milk, 7 g sugar cane, 13 g chocolate powder); b) Traditional Mexican breakfast (TM-B) (≈478 g total weight, 377 Kcal): one scrambled egg, three corn tortillas (90 g), refried beans (83 g), chocolate milk (264 mL whole milk, 6 g sugar cane, 11 g chocolate powder); c) Alternative Mexican breakfast (AM-B) (≈370 g total weight, 308 Kcal): one scrambled egg, two corn tortillas (60 g), chocolate milk (256 mL whole milk, 10 g sugar cane, 18 g chocolate powder). Individual ingredients were purchased from the local market and menus were prepared in the laboratory kitchen according to traditional regional customs. Freshly prepared menus were homogenized in a food processor (NB-101B, Nutribullet, China), frozen (−80 °C), freeze-dried (FreeZone 6, Labconco, USA), ground, sieved through a mesh size of 500 μm, and stored at −20 °C until analysis. Each menu was prepared by triplicate.

2.2. Quantification and isolation of indigestible fraction (IF) in menus

IF was evaluated according to Saura-Calixto, García-Alonso, Goni, and Bravo (2000), a method that simulates the physiological conditions in the upper gastrointestinal tract. The method was run at preparative scale, according to the modifications proposed by Tabernero, Venema, Maathuis, and Saura-Calixto (2011). Insoluble (IIF) was considered as the digestion residues pelleted by centrifugation, while those retained by dialysis represented soluble IF (SIF); the sum of both fractions equals total IF (TIF). TIF was collected, freeze-dried, milled (IKA M20, USA), sieved (500-μm mesh), and stored in seal bags at −20 °C.

2.2.1. Chemical composition of indigestible fraction (IF)

Moisture content, ash, protein, and fat in IF were analyzed according to AOAC (1990) 925.10, 923.03, 920.87 and 920.39 methods, respectively. Indigestible carbohydrates were estimated by Dubois, Gilles, Hamilton, Rebers, and Smith (1956) method; briefly, 250 mg of IF isolated from breakfast menus were suspended with 25 mL of water in constant stirring, 1 mL of this suspension was mixed with 0.5 mL of phenol (5% w/v) and 2.5 mL of concentrated sulfuric acid (36% v/v). The absorbance measured at 480 nm was compared to a standard glucose curve in the range 10–100 μg/mL. Resistant Starch (RS) was evaluated with the methodology proposed by Goñi, García-Diz, Mañas, and Saura-Calixto (1996). Briefly, 25 mg IF were solubilized in 4 M KOH, neutralized with HCl and hydrolyzed with amyloglucosidase (A-9913, Sigma Aldrich, USA, pH 4.75, 60° C). RS was quantified measuring released glucose, using the GOD-POD enzymatic/colorimetric kit (Spinreact, Spain).

2.2.2. Antioxidant compounds and capacity (AOX) analysis in the indigestible fraction

TIF samples (250 mg) from the different menus were extracted with aqueous-organic solution according to the methodology proposed by Pérez-Jiménez, Arranz, and Saura-Calixto (2009). Total soluble polyphenols (TSP) in extractable fraction were determined with the Folin–Ciocalteu's reagent (Montreau, 1972) using a 96-well microplate reader (Biotek, Synergy HT, Winooski VT, USA) with Gen5 software, and the results were expressed as gallic acid equivalents (g GAE/100 g menu FW). Supernatants were evaluated for AOX by1,1-Diphenyl-2-picryl hydrazyl (DPPH) antiradical activity assay was performed according to the method of Prior, Wu, and Schaich (2005). Ferric reducing antioxidant power (FRAP) assay was performed as described by Benzie and Strain (1996). AOX were expressed as mmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) equivalent (TE; mmol/g IF, dry weight, DW).

The residues of the extraction were treated for non-extractable polyphenols quantification, which includes condensed tannins (CT) and hydrolyzable polyphenols (HP). CT were assessed by the method proposed by Reed, McDowell, Van Soest, and Horvath (1982). The residues were treated with 10 mL butanol/HCl (97.5:2.5 v/v) and 0.7 g $FeCl_3$ (3 h, 100 °C) for proanthocyanidin hydrolysis. The absorbance was determined at 555 nm and results were expressed as CT equivalents g/100 g menu FW, using a carob pod (*Ceratonia siliqua*) proanthocyanidin standard. HP were evaluated by Hartzfeld, Forkner, Hunter, and Hagerman (2002) method. The HP values were determined by methanol/H_2SO_4 90:10 (v v-1) hydrolysis of the residues at 85 °C for 20 h. The absorbance was determined at 750 nm and results were expressed as g GAE/100 g menu FW.

2.3. In vitro colonic fermentation by human microflora

The assays were preformed using a pool of fresh fecal samples collected from five healthy schoolchildren (9–12 years), three male (Nutritional status based on body mass index: normal-weight, overweight and obese, respectively) and two female (Nutritional status based on body mass index: normal weight and overweight, respectively), apparently free from gastrointestinal diseases and who did not receive antibiotic treatment during the previous 3 months. Parents signed an assent approving the donation of fecal samples.

The fermentation process was developed according to Campos-Vega et al. (2009). TIF of breakfast menus was fermented at 37 °C under anaerobic conditions. Two different controls were also conducted in parallel: a) raffinose, used as a fermentable sugar reference that produce SCFA, was incubated in the medium with faeces inoculum, and b) the fecal suspension was incubated without addition of substrate, serving as negative control. All incubations were performed in triplicate; samples were collected at 12, 24, 48 and 72 h, and centrifuged (Hermle Z 323 K; Wehingen, Germany) (3500 ×*g*, 15 min, 4 °C). Supernatants were placed into a 20 mL vial sealed with a magnetic cap with a polytetra-fluoro-ethylene (PTFE)/silicon septum. The vials were immediately stored at −80 °C in order to minimize any deteriorating changes in the volatile components of the samples until they were processed.

2.4. Metabolites characterization by HS-SPME-GC/MS

Supernatants from the colonic fermentation were characterized by gas chromatography–mass spectrometry (CGC/MS) using a headspace solid-phase micro-extraction (HS-SPME) according to Zamora-Gasga et al. (2015). The volatile constituents were analyzed with an Agilent 5975C VL mass selective detector coupled to an Agilent 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA), equipped with a DB-5MS capillary column (60 m × 250 μm × 0.25 μm; Agilent). Short chain fatty acids (SCFA) were quantified by means of acetic, propionic, and butyric acid standard curves. Tentative identification of other volatile components was done comparing the mass spectra of the samples with the data system library MSD ChemStation software (Agilent G1701EAversion E.02.00.493). Relative concentration of all fermentation metabolites was calculated using a linear equation from an acetic acid standard curve and the results were expressed in mmol/L.

2.5. Statistical analysis

Data were subjected to one-way ANOVA/Fisher's LSD test (Levene's test, $p \geq 0.05$, Shapiro–Wilk W test, $p \geq 0.05$, $n = 9$) and to independent-samples Kruskal–Wallis non-parametric test/Multiple comparisons of mean ranks for all test groups (Levene's test, $p < 0.05$, Shapiro–Wilk W test, $p < 0.05$, $n = 9$). Two-way ANOVA with a *post-hoc* Fisher's LSD test was used to determine the effect of the substrate type and fermentation time on pH changes and AOX (Substrate type × fermentation time). Principal component analysis (PCA) of fermentation metabolites was performed based on the mean values of triplicates at each specific fermentation time point. Components were calculated without rotation and the number of extracted factors were based on Eigenvalues > 1.0 and explained variance (%) > 70. A secondary analysis based on multiple lineal regression analysis was used to identify which metabolic profiling (Component score) where associated with pH and AOX (DPPH and FRAP methods). In order to identify which samples (blank, raffinose, and IF samples) showed similar metabolic profiles, a hierarchical cluster analysis (HCA) was performed. Single Linkage amalgamation method of clustering and the Euclidean distance measure were used. All analyzes were performed using STATISTICA software, version 10.0 (StatSoft. Inc. 1984–2007, Tulsa, OK, USA).

3. Results and discussion

3.1. Characterization of indigestible fraction (IF) in breakfast menus

Moisture, IF content, and chemical composition of IF menus are shown in Table 1. No significant difference in moisture content were observed between menus, which probably reflects the similar amount of milk included in their preparation (≈250 mL), but significant differences ($p < 0.05$) in TIF content were found between menus (6.11, 6.22 and 5.03 g/100 g FW for MM-B, TM-B, and AM-B respectively). The insoluble IF (IIF) contents in MM-B and TM-B (5.60, 5.13 g/100 g FW) and, TM-B and AM-B (5.13, 4.57 g/100 g FW) were not significantly different. TM-B presented the highest soluble IF (SIF) value (1.10 g/100 g menu FW). This is not surprising since these menus included refried beans which are known as good source of dietary fiber (DF) (Dueñas et al., 2016). Ash content in the IF's showed significant differences ($p < 0.05$), with values ranging from 0.19 to 0.25 g/100 g FW. Different minerals can be poorly absorbed in the human small intestine because of the presence of certain inhibitory compounds like phytates, CT and DF, although they can be absorbed in colon (Raes, Knockaert, Struijs, & Van Camp, 2014). On the other hand, TM-B and MM-B exhibited the highest indigestible carbohydrate content, with 0.89 g/100 g FW. These results could be attributed to the presence of indigestible carbohydrates, from corn tortilla and beans (Sáyago-Ayerdi, Tovar, Zamora-Gasga, & Bello-Pérez, 2014). The indigestible fat content of menus was about 3 g/100 g menu FW, and indigestible protein was about ≈1 g/100 g menu FW, with significant differences ($p < 0.05$) between menus. High concentrations of indigestible protein and low levels of indigestible carbohydrates may have negative implications resulting in decreased colonic production of fecal cancer-protective metabolites (*e.g.* butyric acid) and increased concentrations of hazardous compounds (*e.g. N*-nitroso compounds) (Russell et al., 2011).

Table 1
Chemical composition and antioxidant compounds content, and antioxidant capacity in the indigestible fraction (IF) isolated from breakfast menus[1].

	Menus[2]		
	MM-B	TM-B	AM-B
Moisture content (g/100 g menu)	73.26 ± 0.37a	74.13 ± 0.10a	73.62 ± 0.40[a]
IF (g/100 g menu FW)			
Insoluble IF	5.60 ± 0.22b	5.13 ± 0.21ab	4.57 ± 0.10a
Soluble IF	0.51 ± 0.06a	1.10 ± 0.14b	0.46 ± 0.06a
Total IF[3]	6.11 ± 0.24b	6.22 ± 0.35b	5.03 ± 0.09
IF Nutritional Composition (g/100 g menu FW)			
Ash	0.25 ± 0.01a	0.24 ± 0.01a	0.19 ± 0.01b
Indigestible Carbohydrates[5]	0.73 ± 0.01a	0.89 ± 0.07a	0.34 ± 0.05b
Resistant Starch	0.008 ± 0.001a	0.012 ± 0.001b	0.004 ± 0.001c
Lipids	3.30 ± 0.04a	3.32 ± 0.18a	3.24 ± 0.04a
Proteins[4]	1.30 ± 0.05a	1.42 ± 0.09a	1.07 ± 0.02b
IF Antioxidant Compounds (g/100 g menu FW)			
Total soluble polyphenols	0.07 ± 0.002b	0.06 ± 0.001a	0.06 ± 0.001a
Condensed Tannins	0.04 ± 0.001a	0.13 ± 0.008b	0.05 ± 0.002a
Hydrolyzable polyphenols	0.10 ± 0.001b	0.13 ± 0.001c	0.09 ± 0.001a
IF Antioxidant Capacity (mmol TE/100 g menu FW)			
DPPH	ND	ND	ND
FRAP	485.67 ± 28.34b	404.30 ± 3.73a	381.16 ± 3.04a

[1] Values are mean ± standard error ($n = 3$); fresh weight (FW); Not Detected (ND). Means in rows marked with different letters indicate significant difference ($p < 0.05$).
[2] Menus; MM-B: Modified Mexican Breakfast, TM-B: Traditional Mexican Breakfast, AM-B: Alternative Mexican Breakfast.
[3] Total IF = Sum of soluble IF + insoluble IF.
[4] Conversion factor: %N × 6.25.
[5] Estimated value by the phenol-sulfuric method (Dubois et al., 1956).

3.2. Antioxidant compounds and capacity (AOX) in the indigestible fraction

The levels of TSP, HP, and CT present in IF of the studied menus are summarized in Table 1. TSP associated to IF were released in the aqueous-organic extraction system and showed AOX, but not for DPPH radical scavenging activity was observed (Table 1). TM-B showed the highest CT and HP contents (0.13 g/100 g menu FW and 0.13 g/100 g menu FW, respectively) among the studied breakfasts. The presence of beans in TM-B could be largely responsible for the elevated non-extractable polyphenols content (Ramírez-Jiménez, Reynoso-Camacho, Tejero, León-Galván, & Loarca-Piña, 2015). Also, interactions between indigestible protein and polyphenols occurring in the food matrix may hamper the absorption of polyphenols in the small intestine, increasing their presence in the IF (Mullen et al., 2009). Chelating activity was higher (485.67 mmol TE/100 g menu FW) in extracts from IF isolated from MM-B menus than in those isolated from M-B and AM-B (404 and 381 mmol TE/100 g menu FW, respectively). Besides polyphenols, other constituents present in IF from the breakfasts may affect AOX. The presence of egg yolk components, like phosvitin, phospholipids, carotenoids and free aromatic amino acids, may contribute to the overall AOX of the samples (Remanan & Wu, 2014); however, the concentration of these compounds in IF from food blends has not been studied.

3.3. Changes in pH and antioxidant capacity (AOX) during in vitro colonic fermentation

The progress of the fermentation process was followed measuring pH changes in each sample during 72 h (Fig. 1a). Significant differences

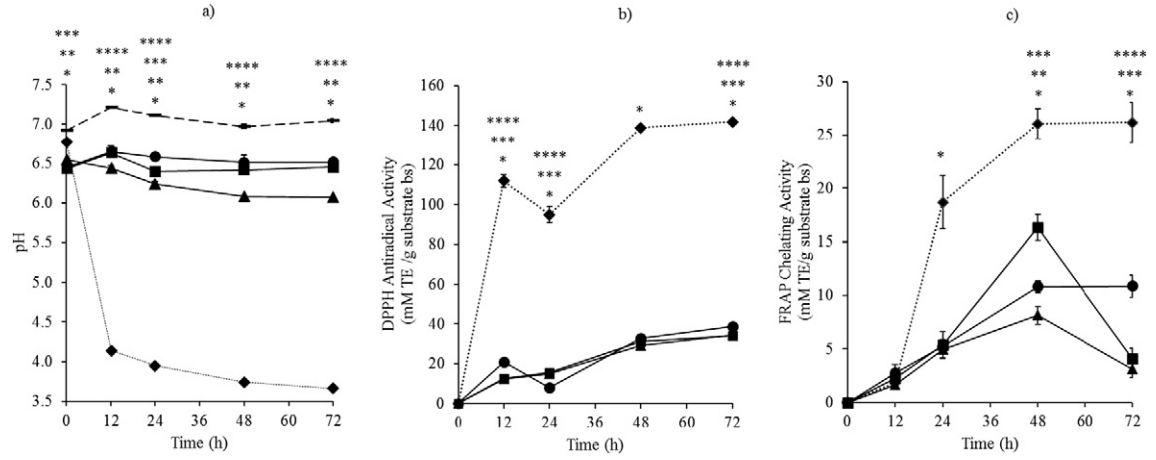


Fig. 1. Changes in a) pH kinetic plot, b) DPPH Antiradical activity plot and c) FRAP chelating activity plot in the extracts during *in vitro* colonic fermentation from (–) blank, (-♦-) raffinose, and indigestible fraction isolated from breakfast menus: (-■-) Modified Mexican Breakfast, MM-B, (-▲-) Traditional Mexican Breakfast, TM-B, and (-●-) Alternative Mexican Breakfast, AM-B at different fermentation times, Values are means ± SEM ($n = 3$). For DPPH and FRAP, blank was subtracted of the samples. *Significant difference between Controls (Raffinose or Blank) and Breakfast menus (MM-B, TM-B and AM-B) **Significant difference between MM-B and TM-B. ***Significant difference between MM-B and AM-B. ****Significant difference between TM-B and AM-B. Significant difference using Two-way ANOVA/Fisher's LSD test (Samples × Time interaction, $p < 0.05$).

($p < 0.05$) were found among the various substrates. The largest decrease in pH was recorded for the raffinose reference, but among the IF from breakfast menus TM-B resulted in the most marked decrease after 72 h (pH = 6.07). The pH differential (pH at 0 h minus pH at 72 h) was −0.02, 0.48, and −0.07 for MM-B, TM-B, and AM-B respectively, indicating that only the IF from TM-B menu showed a decrease in pH (beneficial effect). The pH increase registered for in MM-B and AM-B IFs may be related to poor or limited fermentation of the indigestible carbohydrates present in those substrates (Kettle, Louis, Holtrop, Duncan, & Flint, 2015). AOX evaluated by DPPH and FRAP assays (Fig. 1b and c), showed time-dependence and significant differences ($p < 0.05$). It is noteworthy that DPPH activity (<40 mmol TE/g IF) was detected in fermentation extracts but not in aqueous-organic extracts. In terms of FRAP, MM-B (16.31 mmol TE/g IF DW) showed significant differences from the other two menus at 48 h. However, the AM-B menu had the highest FRAP activity at 72 h (10.85 mmol TE/g IF DW). Microbial metabolites can influence the overall AOX in fermentation extracts (Wang et al., 2015).

3.4. Production of short chain fatty acids (SCFA) during in vitro fermentation

The SCFA production at 12, 24, 48 and 72 h of *in vitro* fermentation of IF isolated from breakfast menus is shown in Table 2. At 24 h, AM-B produced the greatest acetate concentration (47.23 mmol/L), being statistically different ($p < 0.05$) from the other breakfast menus. Propionic acid concentrations were similar ($p > 0.05$) between MM-B and TM-B (28.5 and 28.9 mmol/L respectively), although AM-B showed the highest concentration (35.7 mmol/L) at 24 h. Acetic and propionic acids are related with hepatic cholesterol synthesis, anti-inflammatory properties and satiety effects (Vipperla & O'Keefe, 2012). Regarding butyric acid, the amount produced from the breakfast menus were similar ($p > 0.05$) to that recorded for the blank. Such a low production of SCFA by the IF from the breakfast menus may be related to the macronutrient composition in these preparations. Also, a recent study suggested that the magnitude of SCFA production during *in vitro* fermentation can be dependent on the diet of the fecal donor (Yang & Rose, 2016).

Table 2
Short-chain fatty acids (SCFAs, mmol/L production at 12, 24, 48 and 72 h of *in vitro* fermentation of blank, raffinose and indigestible fraction (IF) isolated from breakfast menus (MM-B; Modified Mexican Breakfast, TM-B; Traditional Mexican Breakfast, AM-B; Alternative Mexican Breakfast)[1].

	IF-breakfast menus				
SCFA/fermentation time	Blank	Raffinose	MM-M	TM-M	AM-M
Acetic acid					
12 h	6.05 ± 0.75a	205.05 ± 25b	2.39 ± 0.08a	12.93 ± 0.69a	6.41 ± 0.87a
24 h	ND	273.92 ± 33.41c	19.88 ± 2.27a	7.45 ± 0.07a	47.23 ± 0.30b
48 h	11.28 ± 6.22a	572.54 ± 24.63b	19.89 ± 2.13a	5.89 ± 0.36a	11.63 ± 1.07a
72 h	ND	254.75 ± 12.48b	20.32 ± 8.69a	ND	17.40 ± 6.22a
Propionic acid					
12 h	4.18 ± 0.62a	44.17 ± 0.89b	ND	ND	ND
24 h	ND	89.01 ± 2.61c	28.50 ± 3.15a	28.91 ± 1.13a	35.76 ± 2.30b
48 h	ND	134.33 ± 8.10c	27.13 ± 1.73b	ND	18.38 ± 0.45a
72 h	ND	103.88 ± 2.46	ND	ND	ND
Butyric acid					
12 h	0.78 ± 0.17a	25.97 ± 5.63b	0.75 ± 0.13a	1.52 ± 0.11a	0.67 ± 0.12a
24 h	0.44 ± 0.09a	36.94 ± 7.92b	3.41 ± 1.00a	4.79 ± 0.24a	4.57 ± 0.30a
48 h	0.78 ± 0.18a	63.55 ± 7.57b	3.49 ± 0.29a	1.85 ± 0.24a	3.86 ± 0.76a
72 h	ND	34.92 ± 1.32	ND	ND	ND

[1] The values are reported in mmol/L produced per 100 mg substrate as mean ± SEM of three replicates; different lowercase letters indicate significant differences in rows among substrates for a time ($p < 0.05$).

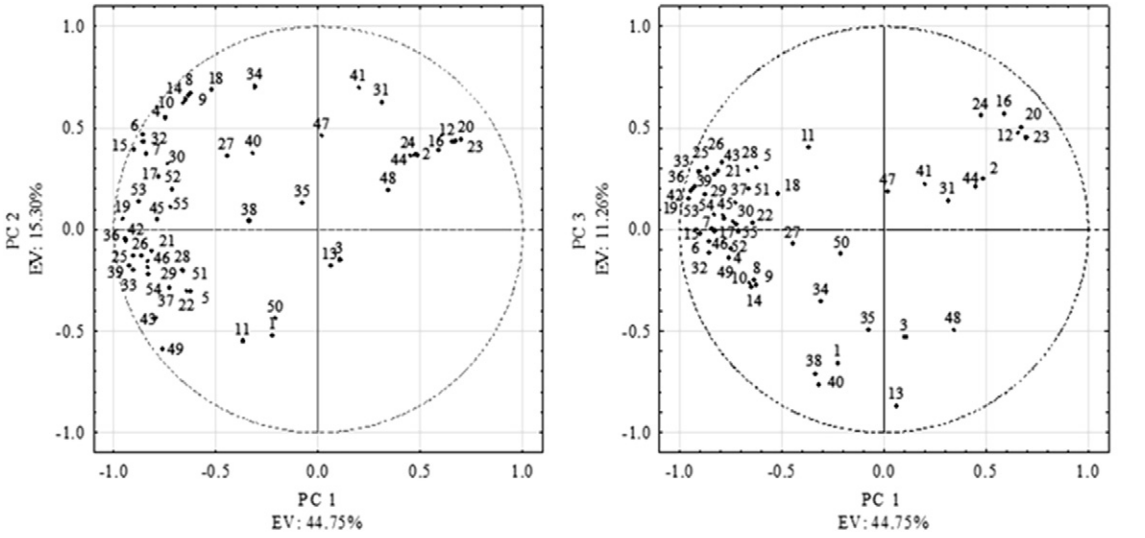
Interestingly, the menu with the lowest indigestible carbohydrate content AM-B) showed the highest production of SCFA. It is thus tempting to suggest that other metabolites may be involved in the pH decrease observed during fermentation of IF from TM-B menu. In this sense, volatile organic compounds were produced during the fermentation of the Ifs (see Table SI, Electronic Supplementary Material, ESM-I). A greater relative concentration of hexanoic acid (ID: 31), heptanoic acid (ID: 34) and octanoic acid (ID: 41) was recorded in the IF fermentation medium from TM-B compared to the other breakfast menus, particularly after 48 and 72 h. The decrease in SCFA concentration at 72 h, is likely due to the ability of some species of bacteria to use metabolites produced by other species as substrates for their own fermentative processes (cross-feeding) (Rios-Covian, Gueimonde, Duncan, Flint, & Clara, 2015). Therefore, reduced pH values may be attributed to the production of non-SCFA organic acids (Sagar, Cree, Covington, & Arasaradnam, 2015).

3.5. Volatile compounds during the in vitro colonic fermentation

A total of 55 volatile compounds were detected in fermentation extracts of blank samples, raffinose standard and IFs isolated from the different breakfast menus (See, ESM-I). PCA was performed to determine microbial metabolic pattern of fermentation in the samples at different fermentation times. Three principal components (PC) were obtained (Eigenvalues > 1) that explained 71.31% of the total variance. Loading scatter plot for the three principal components are shown in Fig. 2. PC 1, 2, and 3 explained 44.75, 15.30, and 11.2%, respectively, of all variance from the gut metabolites production. PC1 on the positive axis (Fig. 2a) was highly influenced by different microbial metabolites, mainly SCFA (See, ESM-I); butyric acid, benzaldehyde, 4-methyl-acetic acid, propionic acid, 3-methyl-, butanoic acid and ethanol (ID: 20, 23, 12, 16, 24, 2). Increased concentrations of these compounds registered during *in vitro* colonic fermentation have been suggested as health-beneficial in the large intestine (Sagar et al., 2015). On the other hand, PC1 negative axis comprises compounds that were produced at relatively low levels during the fermentation process, mainly medium to long chain fatty acid esters (see ESM-I: ID: 5, 19, 21, 25, 36, 39, 46, 49). Regarding PC2 (Fig. 2a), fatty acid esters with less than eight carbon atoms: butanoic acid methyl ester, hexanoic acid methyl ester, hexanoic acid ethyl ester, heptanoic acid methyl ester, heptanoic acid ethyl ester (ID: 4, 8, 9, 10, 14) as well as hexanoic, heptanoic and octanoic acids (ID: 31, 34, 35), were located in the positive axis (high production). Volatile compounds placed on the negative axis (low production) were trimethylamine, dimethyl trisulfide and indole (ID: 1, 11, and 49 respectively). In PC 3 (Fig. 2b), increased production is noticed for propionic acid, butyric acid, and 3-methyl butanoic acid, (ID: 16, 20, 24), while trimethylamine, trichloromethane, 2,4,6-trimethyl-pyridine-phenol, and 4-methyl-phenol (ID:1, 3, 13, 40) were produced in low concentrations.

3.6. Correlation analysis between microbial metabolic pattern, pH and antioxidant capacity

Multiple regression analysis was conducted to test the relationship between microbial metabolic pattern (component scores), pH, DPPH, and FRAP in fermentation extracts of IF isolated from breakfast menus at different times (Fig. 3). The linear regression model was significant ($p < 0.05$) with high values in the coefficients determined, indicating a proper fit of the data with the model (Multiple R^2 values = 0.91, 0.88, and 0.62 for pH, DPPH, and FRAP, respectively). The results showed that the three components were significantly ($p < 0.05$) associated with the decrease in pH values (Fig. 3a). The largest predicted decrease in pH was observed with the fermentative metabolic pattern characterized by a high production of fatty acid esters and volatile organic acids,



ID	Volatile Metabolite	ID	Volatile Metabolite	ID	Volatile Metabolite	ID	Volatile Metabolite
1	Trimethylamine	15	Octanoic acid, methyl ester	29	Undecanoic acid, methyl ester	43	Methyl myristoleate
2	Ethanol	16	Propionic Acid	30	Decanoic acid, propyl ester	44	Nonanoic acid
3	Trichloromethane	17	Octanoic acid, ethyl ester	31	Hexanoic acid	45	Methyl 13-methyltetradecanoate
4	Butanoic acid, methyl ester	18	Benzene, 1,3-bis(1,1-dimethylethyl)-	32	Benzenepropanoic acid, methyl ester	46	Pentadecanoic acid, methyl ester
5	Disulfide, dimethyl	19	Nonanoic acid, methyl ester	33	Dodecanoic acid, methyl ester	47	n-Decanoic acid
6	Pentanoic acid, methyl ester	20	Butyric Acid	34	Heptanoic acid	48	Phenol, 2,4-bis(1,1-dimethylethyl)
7	Pentanoic acid, ethyl ester	21	Nonanoic acid, ethyl ester	35	Benzenemethanol, 4-methyl-	49	Indole
8	Hexanoic acid, methyl ester	22	Acetophenone	36	Dodecanoic acid, ethyl ester	50	9-Hexadecenoic acid, methyl ester, (Z)-
9	Hexanoic acid, ethyl ester	23	Benzaldehyde, 4-methyl-	37	Methyl 10-methyl-dodecanoate	51	Methyl hexadec-9-enoate
10	Heptanoic acid, methyl ester	24	Butanoic acid, 3-methyl-	38	Phenol	52	Hexadecanoic acid, methyl ester
11	Dimethyl trisulfide	25	Decanoic acid, methyl ester	39	Tridecanoic acid, methyl ester	53	Hexadecanoic acid, ethyl ester
12	Acetic Acid	26	Decanoic acid, ethyl ester	40	Phenol, 4-methyl-	54	9-Octadecenoic acid, methyl ester, (E)-
13	Pyridine, 2,4,6-trimethyl-	27	Pentanoic acid	41	Octanoic Acid	55	Ethyl Oleate
14	Heptanoic acid, ethyl ester	28	Methyl 8-methyl-decanoate	42	Methyl tetradecanoate		

Fig. 2. Identification of Microbial metabolic pattern between the *in vitro* colonic fermentation extracts of blank, raffinose and indigestible fraction isolated from breakfast menus at different fermentation times using principal component analysis (PCA): Loading scatter plot for a) PC1 *vs.* PC2; b) PC3 *vs.* PC4 and PC5 *vs.* PC6.

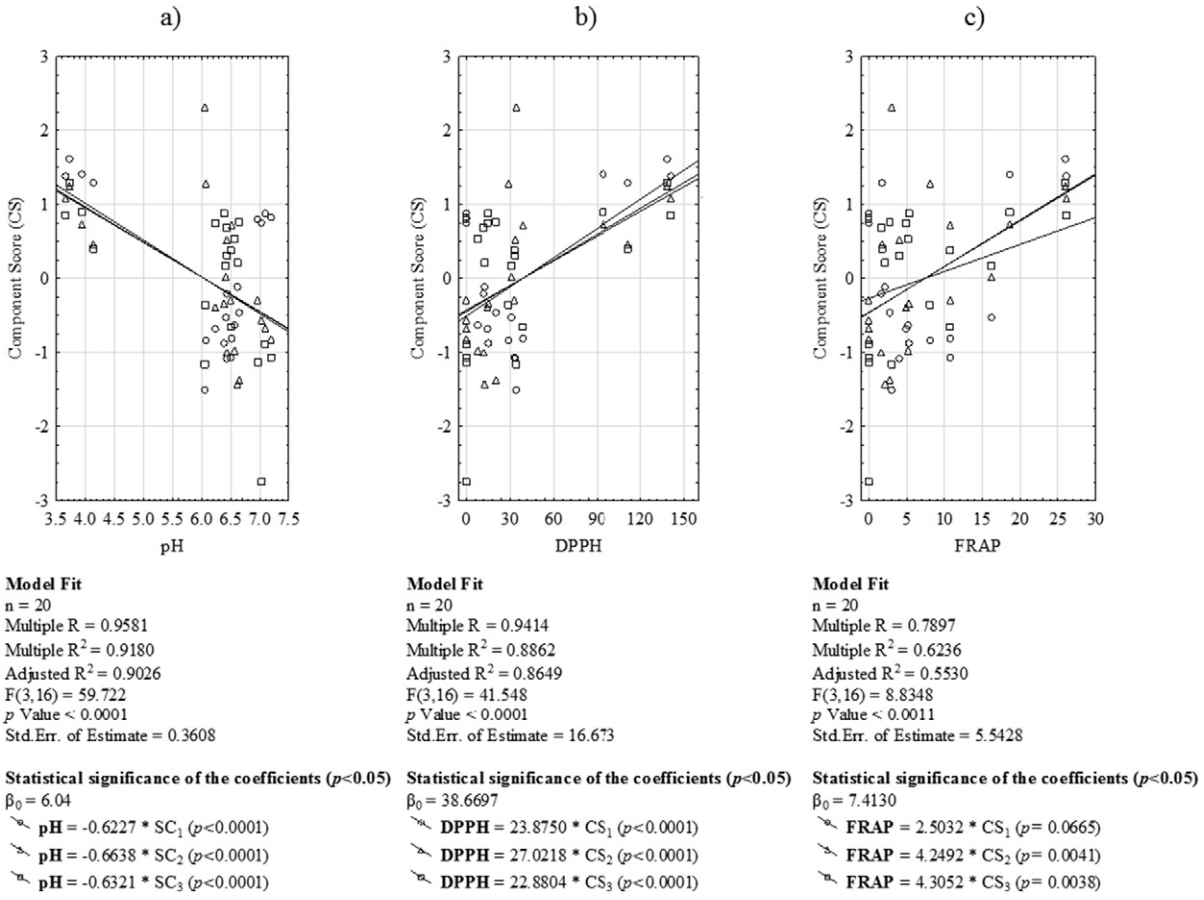


Fig. 3. Multiple regression analysis models exploring the association of microbial metabolic pattern (Component scores) with: a) pH Values, b) DPPH antiradical activity and c) FRAP chelating activity. *Model fit was adjusted to: $Y = \beta_0 + \beta_1 * X_1 + \beta_2 * X_2 + \beta_3 * X_3$, where, Y is response variable (pH, DPPH and FRAP), β_0 is the intercept, $\beta_1, \beta_2, \beta_3$, are the coefficients and X_1, X_2, X_3 are the Component Score (CS_1, CS_2 and CS_3).

with a low production of trimethylamine, dimethyl trisulfide and indole (PC 2). Also, PC3 scores were associated with increased AOX; increase in predicted DPPH (Fig. 3b) was observed with increasing production of PC 2 members (Coefficient = 27.02), followed by production of metabolites included in PC1 (high SCFA production and low fatty acid esters production, Coefficient = 23.87) and PC3 (high propionic and butyric production and low production of protein metabolites, Coefficient = 22.88). Finally, for chelating activity, increased FRAP values (Fig. 3c) were observed with increased scores of PC2 and PC3, while PC1 coefficient was not significant ($p > 0.05$). These results suggest that the overall metabolite composition of the luminal medium may affect both the pH and antioxidant status of the colon. They also highlight the importance of assessing the microbial metabolic pattern instead of individual metabolites identified during IF fermentation isolated from complete foods. Actually, volatile compounds in urine, particularly 2-propanol and acetamide, have been proposed as a potential noninvasive biomarkers that may reflect gut dysbiosis, thus having the capability to differenciate subjects in health and disease (Covington et al., 2013). Additionally, some compounds identified in this work have been previously reported as harmful to health. For instance, experimental and clinical studies have shown trimethylamine-*N*-oxide (formed in the liver from trimethylamine), phenol and *p*-cresol as strong predictors of coronary artery disease and Crohn's disease (Trøseid et al., 2015; Walton et al., 2013).

3.7. Hierarchical cluster analysis (HCA)

Dendrogram of component scores (metabolic profile) from different fermentation times of IF isolated from breakfast menus are presented in Fig. 4. The HCA led to three clusters, indicating that the production of volatile compounds by gut microbiota was different between the formed clusters. The analysis clearly group raffinose (Cluster I) and blank samples (Cluster III) at all times. In addition, Cluster I presented component positive scores for PC 1, indicating that the cluster follows a metabolic profile characterized by high SCFA production and low fatty acid esters (medium to long chain) generation. Cluster II was formed by fermentation extracts of IF isolated from all breakfast menus. However, it is noteworthy that TM-M (at 48 and 72 h) showed the highest values of the scores for PC2 (high production of C < 8 fatty acid esters and volatile organic acids). PC2 was associated with decreased pH and increased AOX. Additionally, the remaining samples were characterized by light colors, indicating that they are not associated with health benefits. Overall, HCA indicated that *in vitro* colonic fermentation varies considerably in the metabolic product pattern depending on the IF sample and fermentation time.

4. Conclusions

This study shows that IFs isolated from the three breakfast menus were different. TM-B contains non-extractable polyphenols, and indigestible carbohydrates (DF). It is being increasingly shown that the fermentative metabolism of ingested food creates a microbial metabolome that has marked impact on the host health. In this work, PCA allowed the identification of metabolic patterns that are associated with beneficial health effects (decreased colonic pH and increased AOX). Compounds associated with different diseases were also identified and associated with harmful effects (increased pH and decreased AOX). HCA showed that fermentation extracts of IF isolated from a TM-B

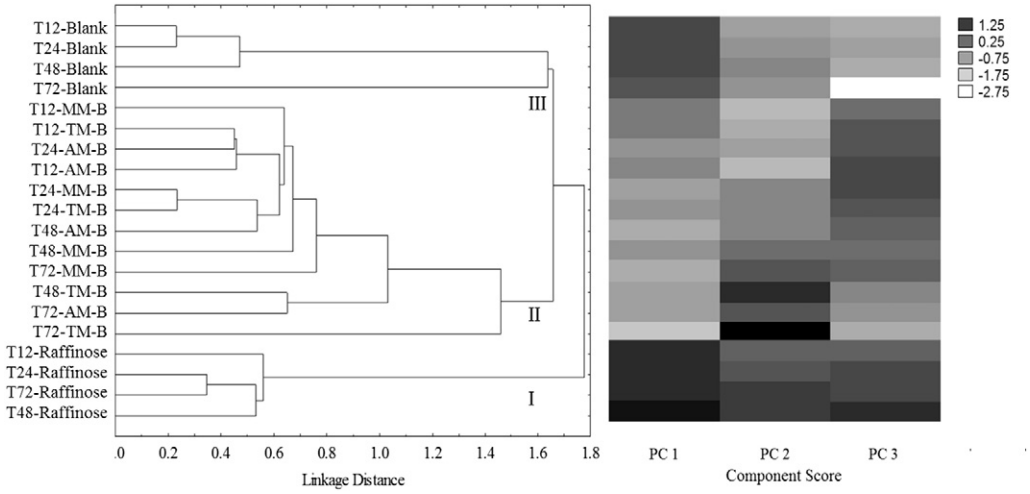


Fig. 4. Dendrogram of hierarchical cluster analysis based on the microbial metabolic profiles (component score) in colonic fermentation extracts of blank, raffinose, and indigestible fraction isolated from breakfast menus (MM-B; Modified Mexican Breakfast, TM-B; Traditional Mexican Breakfast, AM-B; Alternative Mexican Breakfast) at different fermentation times: 12 h (T12), 24 h (T24), 48 h (T48) and 72 h (T72).

presents a time-dependent beneficial metabolic pattern. Further work evaluating the colonic fermentative patterns of foods combined in complete realistic regimens are needed to understand the impact of diet on intestinal and general health status.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.03.038.

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